The formol titration for the determination of ε-amino groups substituted in modified collagens

In the course of an investigation on the crosslinking and modification of collagen it was necessary to devise some method of assessing the extent of the interaction of the various compounds with the amino groups of the protein. Recently COBBETT, GIBBS AND LEACH[1] and LEACH[2] have described the use of the formol titration and a procedure based on reaction with ninhydrin for the examination of modified gelatins. The application of such methods to fibrous proteins, however, is limited by problems related to insolubility such as accessibility to reagents and the attainment of equilibrium.

Two approaches have yielded useful results, amino acid analysis and a modified formol titration. The latter has the advantage that it is carried out under relatively mild conditions unlikely to affect the modified protein and that problems associated with accessibility of the reactive groups of the protein are reduced by the small size of the formaldehyde molecule.

The difficulties of attaining equilibrium in a two-phase system have been reduced by the use of a titrator of the pH stat type (Radiometer TTTlc) and the addition of NaCl to regulate swelling. Examination of the titration curves of collagen in the presence of NaCl (ref. 3) indicated that initial adjustment of the pH to 9.0 followed by titration back to the same pH value should give a satisfactory estimate of the number of ε-amino groups.

After some preliminary experiments the following procedure was adopted. The protein was ground to pass 26 mesh and a sample (usually 2 g) was suspended in 100 ml

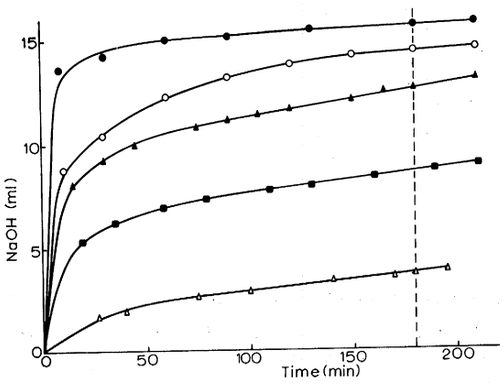

Fig. 1. Formol titration of collagen and modified collagen. 2 g powdered collagen suspended in 100 ml 4% (w/v) NaCl, pH adjusted to 9.0 over 3 h, formaldehyde added and suspension titrated to pH 9.0 by addition of 0.04 M NaOH. ○—○, collagen; ●—●, denatured collagen; modified collagens: ▲—▲, glyoxal; △—△, glutaraldehyde; ■—■, phenyl isocyanate.

4% (w/v) NaCl in a suitable titration vessel. After allowing the sample to wet back thoroughly the vessel was connected to the Radiometer assembly and the pH brought to 9.0 by the addition of 0.04 M NaOH also containing 4% (w/v) NaCl. Commercial Formalin solution (40%, w/v, formaldehyde) previously adjusted to pH 9.0 was then added to give a final concentration of not less than 0.8 M after titration and the pH again brought to 9.0. A blank titration of the formaldehyde–salt solution without protein was also carried out.

Typical curves showing addition of alkali plotted against time are shown in Fig. 1. In all cases there is rapid addition of alkali in the initial stages which gradually slows off and approaches a steady rate after 2–3 h. This slow titration continues for periods of up to 24 h and is apparent in both stages of the titration, *i.e.* before and after the addition of formaldehyde. It is only very slightly reduced by titration in an inert atmosphere and, therefore, is not primarily associated with absorption of CO_2 or other atmospheric contaminants. Slow hydrolysis of amide groups was considered as a possible cause but no evidence for release of ammonia during titration could be obtained. In view of the recent findings of BAKERMAN AND HARTMAN[4,5] concerning titration of amino groups in soluble collagens, it seems most likely that the continued titration is due to slow attainment of equilibrium associated with reduced accessibility of some of the amino groups.

With a purified collagen preparation obtained from ox hide, titration for 3 h gave values between 29 and 31 mmoles ε-amino groups per 100 g compared with a figure of 33 mmoles/100 g indicated by amino acid analysis. Denaturation for 4 h at 65° caused an increase to 33–34 mmoles/100 g indicating increased accessibility of amino groups. However, the increase might well be related to breakdown rather than stereochemical effects.

TABLE I

AMINO GROUPS SUBSTITUTED (mmoles/100 g)

Modifying agent	*From formol titration*	*From amino acid analysis*
Glyoxal	44	0
Glutaraldehyde	25	26
Acrylonitrile	7	9
Phenylisocyanate	15	—
Benzene sulphonyl chloride	8	10
Difluoro-dinitrodiphenyl sulphone	18	19
Cyanuric chloride	22	7

Although equilibrium is not completely attained the titration has proved useful for the assessment of the degree of substitution of amino groups in modification studies[6]. By subtracting the value obtained for free amino groups in the modified collagen from the corresponding value obtained for the original collagen many of the errors inherent in the method are eliminated. Curves for some modified collagens are given in Fig. 1 and results calculated in terms of mmoles/100 g moisture-free collagen in Table I. No allowance is made here for dilution of the protein by the bound compound. Cor-

rection by the procedure suggested by LEACH[2] is not applicable with bifunctional compounds since one or two amino groups may be combined with one molecule of reactant or where polymerisation may have occurred. In such cases results are better expressed on a protein basis calculated from nitrogen determinations or, where this is impossible, hydroxyproline.

Amino acid analyses can also give useful information on the degree of substitution of amino groups. Losses of lysine and hydroxylysine run parallel with substitution of amino groups as estimated by the formol titration in many cases, *e.g.* glutaraldehyde, succinaldehyde, acrylonitrile, benzene sulphonyl chloride and difluorodinitrodiphenyl sulphone. With formaldehyde, glyoxal, cyanuric chloride and its derivatives, however, there is complete or partial release of lysine during hydrolysis.

The method is presumably applicable to other insoluble proteins, *e.g.* elastin and keratin.

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1 W. G. COBBETT, J. A. GIBBS AND A. A. LEACH, *J. Appl. Chem.*, 14 (1964) 296.
2 A. A. LEACH, *Biochem. J.*, 98 (1966) 506.
3 J. H. BOWES AND R. H. KENTEN, *Biochem. J.*, 43 (1948) 358.
4 B. K. HARTMAN AND S. BAKERMAN, *Biochemistry*, 5 (1966) 2221.
5 S. BAKERMAN AND B. K. HARTMAN, *Biochemistry*, 5 (1966) 3488.
6 J. H. BOWES AND C. W. CATER, *Biochim. Biophys. Acta*, 168 (1968) 341.